Cell, Vol. 91, 521–530, November 14, 1997, Copyright ©1997 by Cell Press

Identification and Molecular Characterization of Fractalkine Receptor CX_3CR1, which Mediates Both Leukocyte Migration and Adhesion

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Summary

Leukocyte trafficking at the endothelium requires both cellular adhesion molecules and chemotactic factors. Fractalkine, a novel transmembrane molecule with a CX_3C-motif chemokine domain atop a mucin stalk, induces both adhesion and migration of leukocytes. Here we identify a seven-transmembrane high-affinity receptor for fractalkine and show that it mediates both the adhesive and migratory functions of fractalkine. The receptor, now termed CX_3CR1, requires pertussis toxin–sensitive G protein signaling to induce migration but not to support adhesion, which also occurs without other adhesion molecules but requires the architecture of a chemokine domain atop the mucin stalk. Natural killer cells predominantly express CX_3CR1 and respond to fractalkine in both migration and adhesion. Thus, fractalkine and CX_3CR1 represent new types of leukocyte trafficking regulators, performing both adhesive and chemotactic functions.

Introduction

Blood leukocytes rapidly emigrate into sites of injury or infection, while mature lymphocytes continually recirculate between blood and tissues. These migratory properties of blood leukocytes and circulating lymphocytes are essential for host defense and immunological surveillance. The molecular control of these essential trafficking events requires two broad classes of molecules: various cell adhesion molecules, especially those of selectins and integrins, as well as leukocyte chemotactic factors including the members of the chemokine superfamily (Butcher, 1991; Springer, 1994; Butcher and Picker, 1996). Chemokines have been postulated to work in concert with adhesion molecules in selective trafficking of specific leukocyte classes and lymphocyte subsets, yet many of the molecular workings of these processes remain to be elucidated.

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Chemokines are a group of small, structurally related, and frequently heparin-binding cytokines that provide important signals for leukocyte migration through induction of cell motility and activation of adhesion molecules (Baggiolini et al., 1994; Schall and Bacon, 1994; Rollins, 1997). Chemokines are divided into three major subfamilies, CXC, CC, and C, based on the number and spacing of the first two cysteines in a conserved cysteine structural motif. Different chemokine classes tend to exhibit different ranges of leukocyte specificity: the CXC molecules seem biased in targeting neutrophils and to a lesser extent lymphocytes; the CC molecules mainly target monocytes, and also lymphocytes, basophils, and eosinophils, with varying selectivity; and the C chemokine appears to act preferentially on lymphocytes (Baggiolini et al., 1994; Schall and Bacon, 1994; Rollins, 1997). Chemokine receptors identified to date on leukocytes all manifest a seven transmembrane (7-TM) G protein–linked architecture (Murphy, 1994; Premack and Schall, 1996). It is known that they transduce signals that lead to cytoskeletal reorganization, integrin activation, and other functions leading to increased adhesion and migration of the cells. These effects are frequently mediated through pertussis toxin (PT)–sensitive G protein–coupled pathways.

Recently, a novel fourth class of chemokine, fractalkine, has been identified and characterized (Bazan et al., 1997). Fractalkine is structurally distinct from other types of chemokines. This molecule contains a unique CX_3C motif and exists as a membrane-bound glycoprotein with the chemokine domain atop an extended mucin-like stalk. Membrane-bound fractalkine can be markedly induced on primary endothelial cells by inflammatory cytokines; this form promotes the robust adhesion of monocytes and T lymphocytes. Soluble fractalkine can be released, presumably by proteolysis at a membrane-proximal dibasic cleavage site similar to those of syndecans, and exhibits an efficient chemotactic activity for monocytes and T cells. Thus, fractalkine is a versatile molecule regulating both cell–cell interactions as the membrane-bound form and directed cell migration by its soluble form.

The novel structure and functions of fractalkine suggest that it may play a key role in the multistep process of leukocyte trafficking, while raising several issues. It is not known whether fractalkine induces both its migratory and adhesive effects directly and whether it functions through a standard 7-TM chemokine receptor, an adhesion counter receptor, or a combination of both. We set out to address these questions first by identifying the fractalkine receptor and then to use this information to dissect the molecular functions of the receptor-ligand pair. We report here the identification of a high-affinity functional receptor for fractalkine, CX_3CR1, and how fractalkine and CX_3CR1 directly mediate the adhesion and migration of leukocytes such as monocytes and NK cells. Our findings suggest that fractalkine and CX_3CR1 represent a new class of leukocyte trafficking regulator, where the hybrid functions of both adhesion and migration are combined at molecular and functional levels.

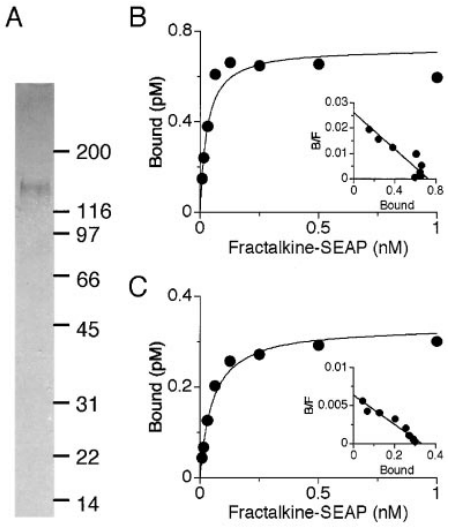

Figure 1. High-Affinity Binding of Soluble Fractalkine-SEAP to Fresh Peripheral Blood Lymphocytes and Monocytes

(A) Purified Fractalkine-SEAP fusion protein. Fractalkine-SEAP was purified from culture supernatants of transfected 293/EBNA-1 cells by a metal affinity chromatography. After electrophoresis on a 4%–20% gradient polyacrylamide gel, proteins were stained with Coomassie brilliant blue. Positions of size markers (kDa) are shown on the right.

(B and C) Saturable binding of Fractalkine-SEAP to lymphocytes and monocytes. Cells (2×10^5 cells) were incubated for 1 hr at 16°C with indicated concentrations of Fractalkine-SEAP. Nonspecific binding was determined by the addition of 200 nM of soluble fractalkine and subtracted. Representative results from three separate experiments are shown. Inserts show Scatchard analysis of the binding data. The calculated K_ds are 30 pM for lymphocytes and 50 pM for monocytes.

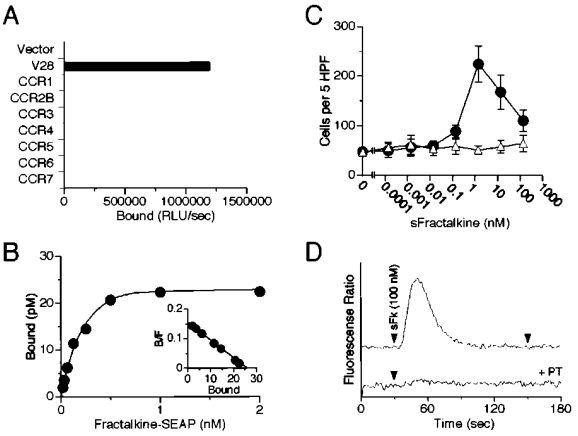

Figure 2. V28 Is a Specific, High-Affinity Receptor for Fractalkine

(A) Specific binding of soluble Fractalkine-SEAP to V28-transfected K562 cells. Cells (2×10^5 cells) were incubated for 1 hr at 16°C with 1 nM of Fractalkine-SEAP. After washing, bound Fractalkine-SEAP was determined enzymatically (Imai et al., 1997). Each histogram represents mean ± range.

(B) Saturable binding of Fractalkine-SEAP to V28-transfected K562 cells. Cells (2×10^5 cells) were incubated for 1 hr at 16°C with indicated concentrations of Fractalkine-SEAP. Nonspecific binding was determined by the addition of 200 nM of soluble fractalkine and subtracted. Representative results from three separate experiments are shown. Insert shows Scatchard analysis of the binding data. The calculated K_d is 100 pM.

(C) Induction of chemotaxis in V28-transfected cells by soluble fractalkine. 293/EBNA-1 cells transfected with V28 (closed circles) or vector alone (open triangles) were tested for migration toward soluble fractalkine at indicated concentrations by using a 48-well chemotaxis chamber. The assay was done in triplicate, and the number of migrating cells in five high-power fields (400×) were counted for each well. Representative results from three separate experiments are shown. Each point represents mean ± SEM.

(D) Calcium mobilization in V28-transfected K562 cells by soluble fractalkine. Cells with or without pretreatment with PT (PT) at 500 ng/ml for 1 hr were loaded with fura-PE3-AM and stimulated with soluble fractalkine (100 nM). Arrowheads indicate time of application of soluble fractalkine. Intracellular concentration of calcium was monitored by fluorescence ratio (F340/F380). Representative results from three separate experiments are shown.

Results

Use of Fractalkine-Alkaline Phosphatase Fusion Protein for Receptor Detection

Previous studies have shown that chemokines fused with the secreted form of placental alkaline phosphatase (SEAP) retain their ability to bind specifically to their cell-surface receptors, while the phosphatase activity allows quantitative determination of specific binding (Luster et al., 1995; Imai et al., 1997). To prepare labeled fractalkine for receptor binding experiments, we expressed soluble fractalkine fused with SEAP (Fractalkine-SEAP). As shown in Figure 1A, purified Fractalkine-SEAP exhibited a broad band with an apparent molecular mass of 150 kDa, consistent with the observed M_r of the soluble form (Bazan et al., 1997) with the added mass of SEAP and $(His)_6$. To characterize fractalkine receptor, we first carried out Fractalkine-SEAP binding experiments using peripheral blood lymphocytes and monocytes known to respond to fractalkine (Bazan et al., 1997). When the binding was performed with increasing concentrations of Fractalkine-SEAP, a single class of receptors was revealed on lymphocytes with a K_d of 30 pM and 440 sites per cell (Figure 1B) and on monocytes with a K_d of 50 pM and 450 sites per cell (Figure 1C). These results clearly demonstrated that Fractalkine-SEAP functions as a high-affinity ligand for the fractalkine receptor, and that lymphocytes and monocytes express a single class of high-affinity receptors for fractalkine.

V28 Is a High-Affinity Functional Receptor for Fractalkine

To identify the fractalkine receptor, we tested the binding of Fractalkine-SEAP to the seven known CC chemokine receptors (CCR1–CCR7) (Baba et al., 1997; Imai et al., 1997; Rollins, 1997; Yoshida et al., 1997) and several "orphan" receptors: BLR1 (Dobner et al., 1992), V28 (Combadiere et al., 1995; Raport et al., 1995), and GPR-9–6 (GenBank accession number: HSU45982). Of the receptors tested, Fractalkine-SEAP bound specifically to V28 expressed on K562 (Figure 2A) as well as on Raji and 293/EBNA-1 (data not shown). When the binding was performed with increasing concentrations of Fractalkine-SEAP, K562-V28 demonstrated a single class of receptors for Fractalkine-SEAP with a K_d of 100 pM and 20,000 sites per cell (Figure 2B). This high-affinity binding of Fractalkine-SEAP to V28 was quite consistent

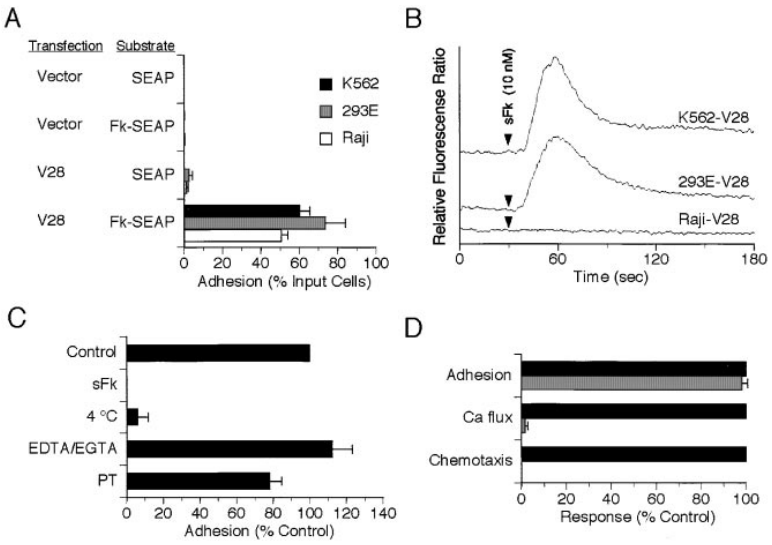

Figure 3. Role of Signaling via V28 in Adhesion to Fractalkine

(A) Specific adhesion to immobilized Fractalkine-SEAP. Cells transfected with vector alone or V28 were applied to wells precoated with SEAP or Fractalkine-SEAP (4000 cells/mm^2) and incubated for 30 min at room temperature. After washing off nonadherent cells by dipping the slides gently in assay buffer twice, adherent cells were fixed with 1% glutaraldehyde and counted in five 400× fields (0.07 mm^2) per well. The data shown represent mean ± SEM from at least three independent experiments. Closed columns, K562; shaded columns, 293/EBNA-1; open columns, Raji.

(B) Calcium mobilization in V28-transfected cells by soluble fractalkine. Cells were loaded with fura-PE3-AM and stimulated with soluble fractalkine (10 nM). Arrowheads indicate time of application of soluble fractalkine. Intracellular concentration of calcium was monitored by fluorescence ratio (F340/F380). Representative results from three separate experiments are shown.

(C) Effect of various treatments on binding of V28-transfected K562 to immobilized Fractalkine-SEAP. For 4°C treatment, adhesion assay was performed at 4°C for 30 min. For soluble fractalkine (sFk) or EDTA/EGTA treatments, 10 μg/ml of soluble fractalkine or 5 mM of EDTA/EGTA was added to the assay buffer during the assay. For treatment with PT, cells were pretreated with PT at 500 ng/ml for 30 min at 37°C and washed before the assay. The data shown represent mean ± SEM of percent adhesion of control (without inhibitor or treatment) from at least three separate experiments.

(D) Effect of PT treatment on fractalkine-mediated adhesion, chemotaxis, and calcium mobilization of V28-transfected 293/EBNA-1. Cells without (closed columns) or with pretreatment with PT at 500 ng/ml for 30 min at 37°C (shaded columns) were assessed for calcium mobilization stimulated with soluble fractalkine (10 nM), chemotaxis response induced by soluble fractalkine (10 nM), and cell adhesion to immobilized Fractalkine-SEAP. The data shown represent mean ± SEM of percent response of control (without PT) from two separate experiments.

with those obtained with the endogenous fractalkine receptors expressed on lymphocytes and monocytes (Figure 1).

We next tested whether V28 was a functional receptor for fractalkine. As shown in Figure 2C, soluble fractalkine induced a vigorous migration of V28-transfected 293/EBNA-1 cells with a typical bell-shaped dose-response curve and a maximum effect at 1 nM. 293/EBNA-1 cells transfected with the vector alone did not respond to fractalkine at all. A checkerboard-type analysis indicated that the migration of V28-transfected 293/EBNA-1 cells toward soluble fractalkine was mostly chemotactic (data not shown). As shown in Figure 2D, fractalkine also induced a vigorous calcium flux in K562 cells expressing V28 with complete desensitization to successive stimulation with fractalkine. No such response to fractalkine was seen in parental K562 cells or those transfected with the vector alone (data not shown). Fractalkine-induced calcium mobilization in K562-V28 cells was detectable at 0.1 nM and reached a maximum level at 10 nM with an EC_{50} of 2 nM (data not shown). Furthermore, pretreatment of K562-V28 cells with PT completely abolished this calcium mobilization (Figure 2D), indicating signal transduction through Gαi-class G proteins. These observations clearly demonstrated that V28 was indeed a functional receptor for fractalkine.

V28 Mediates Cell Adhesion

We next examined whether fractalkine supports adhesion of V28-expressing cells. To minimize involvement of other cell adhesion molecules acting in parallel or in trans, we used glass slides on which Fractalkine-SEAP was immobilized through anti-SEAP antibody. As shown in Figure 3A, K562, 293/EBNA-1, and Raji transfected with V28 efficiently adhered to immobilized Fractalkine-SEAP, but not to SEAP alone. The cells transfected with the vector alone did not adhere to immobilized Fractalkine-SEAP or SEAP at all. Adhesion of V28-transfected cells to immobilized Fractalkine-SEAP appeared to be quite rapid, since most cells were already bound during a few min at room temperature while they were allowed to settle down at 1× g. The adhesion was also quite stable even when cells were incubated at 37°C for 1 hr. This contrasted to the previous observation that adhesion of T cells and monocytes to fractalkine-expressing HEK 293 cells was transient (Bazan et al., 1997). This may suggest adhesion-regulatory activity by these types of cells, such as cleavage of cell surface fractalkine sometime after cell–cell binding. Another notable point was that, in contrast to V28-transfected K562 and 293/EBNA-1 cells, V28-transfected Raji cells did not respond to soluble fractalkine in the calcium mobilization assay at all (Figure 3B). We confirmed that the levels of V28 expression on the cell surface of K562, 293/EBNA-1, and Raji as determined by the Fractalkine-SEAP binding assay (see below) were quite similar (data not shown). This implied that calcium mobilization might not be required for V28-mediated adhesion by fractalkine.

We further investigated the role of signaling via V28 in fractalkine-induced adhesion. As shown in Figure 3C, soluble fractalkine (SFK) was found to suppress completely the adhesion of K562-V28 to immobilized Fractalkine-SEAP. This was striking because soluble fractalkine itself was a potent agonist for V28 in chemotaxis and calcium mobilization (Figure 2). The adhesion was also almost completely suppressed at 4°C. On the other

hand, adhesion of K562-V28 to Fractalkine-SEAP was not affected at all by depletion of divalent cations with EDTA/EGTA. The same treatment was separately confirmed to suppress completely the integrin-mediated cell aggregation of peripheral blood mononuclear cells induced by phorbol myristate acetate (data not shown). Furthermore, PT did not strongly affect the ability of K562-V28 to adhere to immobilized Fractalkine-SEAP, even though the same treatment completely suppressed calcium mobilization in K562-V28 by soluble fractalkine (Figure 2B). We also confirmed that cholera toxin, which is known to inhibit the Gs class of G proteins, did not affect the adhesion either (data not shown). The effect of PT was further examined by using 293/EBNA-1 cells. As shown in Figure 3D, PT had no inhibitory effect on adhesion of V28-expressing 293/EBNA-1 to immobilized Fractalkine-SEAP while it completely inhibited calcium flux and chemotaxis in the same cells induced by soluble fractalkine. Collectively, immobilized Fractalkine-SEAP appeared to induce stable adhesion of V28-expressing cells without involving additional adhesion molecules such as integrins or selectins and without signaling through PT-sensitive G proteins. The temperature dependence of adhesion might indicate involvement of other signaling pathways or necessity of membrane fluidity to allow multiple associations.

Roles of the Chemokine and Mucin Domains in Adhesion

To define the respective roles of the chemokine and mucin domains of fractalkine in adhesion, we next generated three representative mutants: (1) Mucin-SEAP lacking the CX_3C-chemokine domain; (2) CX_3C-SEAP lacking the mucin domain; and (3) TARC-Mucin-SEAP, a chimera where the CX_3C domain was replaced by a CC chemokine TARC (Imai et al., 1997). We first determined the domain responsible for V28 binding. As shown in Figure 4A, CX_3C-SEAP bound to V28-expressing K562 cells as efficiently as Fractalkine-SEAP. Their binding affinity and number of binding sites were equivalent. In contrast, Mucin-SEAP did not bind to V28 at all. These results clearly indicated that the CX_3C domain was solely responsible for binding to V28 while the mucin domain had no direct role. We next examined the role of each fractalkine domain in adhesion of V28-expressing cells. As shown in Figure 4B, immobilized Fractalkine-SEAP (Fk-SEAP), but not those lacking the CX_3C domain (i.e., SEAP, TARC-Mucin-SEAP and Mucin-SEAP), supported adhesion of V28-expressing K562. This confirmed that the CX_3C domain was essential for interaction with V28. Notably, immobilized CX_3C-SEAP lacking the mucin domain failed to support the adhesion of K562-V28 cells, even though soluble CX_3C-SEAP bound to V28 as efficiently as soluble Fractalkine-SEAP (Figure 4A). Thus, the mucin domain appeared to be somehow involved in the adhesion process. The combination of the two mutants (CX_3C-SEAP + Mucin-SEAP), however, failed to support the adhesion in trans. Unexpectedly, when we carried out the same experiments using CCR4-expressing K562 cells (Figure 4C), these cells were found to adhere efficiently to immobilized TARC-Mucin-SEAP. This adhesion was mediated by the specific interaction of CCR4 and TARC (Imai et al., 1997), since K562-CCR4 did not adhere to SEAP, Fractalkine-SEAP, or

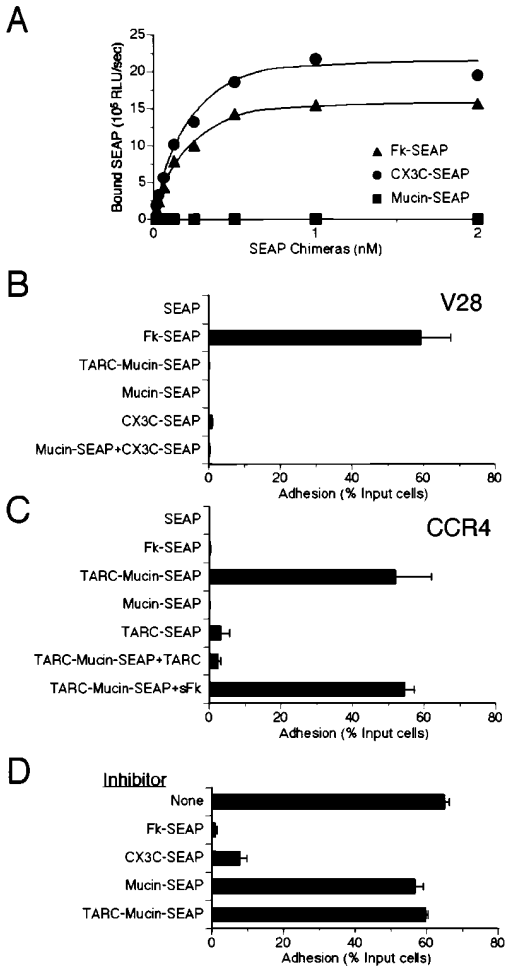

Figure 4. Role of the Chemokine and Mucin Domains in Adhesion

(A) The role of chemokine and mucin domains in binding to V28. V28-transfected K562 cells were incubated for 1 hr at 16°C with indicated concentrations of the SEAP fusion proteins. Nonspecific binding was determined by addition of 200 nM of soluble fractalkine and subtracted. Representative results from three separate experiments are shown.

(B and C) The role of chemokine and mucin domains in adhesion to various immobilized SEAP fusion proteins. The percent adhesion of input cells was determined by counting cells in five 400× fields (0.07 mm^2) per well. The data shown represent mean ± SEM of percent adhesion of input cells from at least two independent experiments.

(D) Inhibitory effect of indicated soluble SEAP fusion proteins on adhesion of V28-transfected K562 cells to immobilized Fractalkine-SEAP. The percent adhesion of input cells was determined by counting cells in five 400× fields (0.07 mm^2) per well.

Mucin-SEAP. Furthermore, TARC, but not soluble fractalkine, inhibited K562-CCR4 adhesion to immobilized TARC-Mucin-SEAP. Another important point was that K562-CCR4 cells again failed to adhere efficiently to immobilized TARC-SEAP lacking the mucin domain.

To further test the respective roles of the chemokine and mucin domains in adhesion, we assessed inhibitory activity of various soluble SEAP fusion proteins on adhesion of K562-V28 cells to immobilized Fractalkine-SEAP. In these experiments, Fractalkine-SEAP was directly immobilized onto glass slides. As shown in Figure 4D,

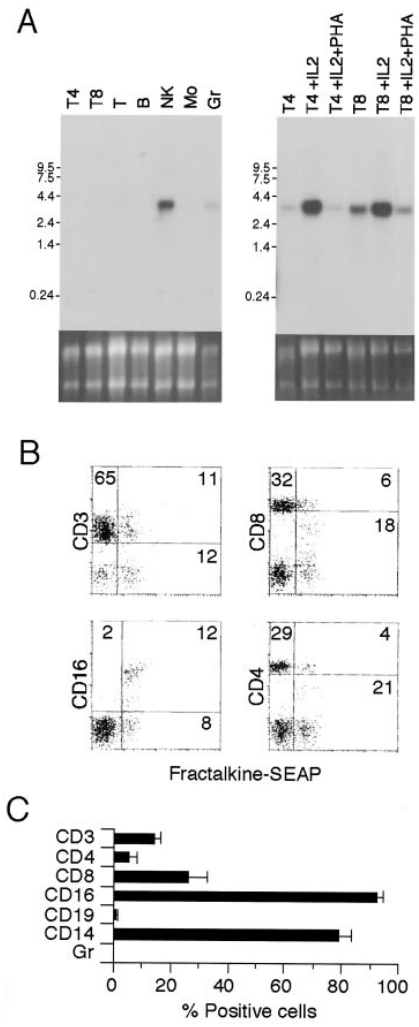

Figure 5. Expression of *V28* mRNA and Surface Fractalkine Receptor in Human Peripheral Blood Leukocyte Populations

(A) Expression of *V28* mRNA. Total RNA samples (5 μg per lane) were subjected to Northern blot analysis by using the ^{32}P-labeled *V28* cDNA as probe. The autoradiograph of the filter (upper) and the photograph of the gel stained with ethidium bromide (lower) are shown. Positions of size markers (kb) are shown on the left. Left panel: T4, $CD4^+$ T cells; T8, $CD8^+$ T cells; T, total T cells; B, B cells; NK, natural killer cells; Mo, monocytes; and Gr, granulocytes. Right panel: fresh $CD4^+$ T cells (T4), $CD8^+$ T cells (T8); and those cultured in the presence of IL-2 (400 U/ml) without or with PHA for 5 days.

(B) Surface expression of fractalkine receptor. PBMC were incubated with SEAP or Fractalkine-SEAP at 4°C for 30 min. After washing, cells were incubated with biotinylated anti-SEAP antibody at 4°C for 30 min. After washing, cells were stained with FITC-streptavidin and PE-labeled antibody for indicated cell surface makers. After washing, cells were analyzed on a FACStar Plus gating for lymphocytes. For $CD14^+$ monocytes, a scatter-gated population was used for analysis. Representative results are shown.

(C) The mean ± SEM from three different donors.

not only soluble Fractalkine-SEAP but also CX_3C-SEAP without the mucin domain strongly suppressed the adhesion of K562-V28 to immobilized Fractalkine-SEAP. On the other hand, Mucin-SEAP or TARC-Mucin-SEAP, both having the mucin domain, had virtually no inhibitory effect on the adhesion. Thus, the mucin domain might not be directly involved in adhesion; the data strongly suggest that the chemokine domain and V28 were solely responsible for direct receptor engagement and adhesion.

Expression of *V28* and Fractalkine Receptor in Leukocyte Classes and Subsets

We next proceeded to determine the expression of *V28* mRNA and surface fractalkine receptor in various types of leukocytes. As shown in Figure 5A, among freshly isolated normal peripheral blood leukocytes, *V28* mRNA was expressed mainly in $CD16^+$ natural killer (NK) cells. After long exposure, low levels of the mRNA were detected in $CD3^+$ T cells, $CD14^+$ monocytes (Mo), and granulocytes (Gr), while $CD19^+$ B cells were virtually negative. Expression of *V28* mRNA in both $CD4^+$ and $CD8^+$ T cells was strongly up-regulated by IL-2 (Figure 5A). The effect of IL-2 was, however, completely suppressed by cotreatment with phytohemagglutinin (PHA). Thus, the expression of V28 in T cells is positively regulated by IL-2 but negatively regulated by T-cell activation like those of CCR1 and CCR2 (Loetscher et al., 1996). To assess surface expression of fractalkine receptor on various leukocyte subsets, cells were incubated with Fractalkine-SEAP and then reacted with biotinylated anti-SEAP antibody. Subsequently, cells were double-stained with FITC-labeled streptavidin and with PE-labeled antibody for indicated surface makers (Figure 5B). As summarized in Figure 5C, most $CD16^+$ NK cells (~92%) were positive for fractalkine receptor. A subpopulation of $CD3^+$ T cells (~14%) also expressed fractalkine receptor. The $CD3^+$ T cells that expressed fractalkine receptor were mostly $CD8^+$ T cells, although some individuals contained both $CD4^+$ and $CD8^+$ T cells expressing fractalkine receptor at similar levels as shown in Figure 5B. Very few $CD19^+$ B cells expressed fractalkine receptor. The surface expression pattern of the fractalkine receptor on lymphocytes thus correlates well with the pattern of *V28* mRNA expression (Figure 5A). The majority of $CD14^+$ monocytes (79%) also expressed fractalkine receptor. On the other hand, virtually no fractalkine receptor was detected on the surface of granulocytes, although *V28* mRNA was present at low levels in these cells (Figure 5A).

Transendothelial Chemotactic Activity of Fractalkine

After demonstrating the expression of *V28* mRNA and surface fractalkine receptor in particular subsets of leukocytes, we examined the phenotypes of cells migrating toward fractalkine by using the transendothelial migration assay (Carr et al., 1994; Oin et al., 1996). Soluble fractalkine induced migration of both lymphocytes and monocytes through endothelial cells with a maximum response at 10 nM (Figure 6A). The efficacy of fractalkine was comparable to that of MCP-1 in lymphocyte chemotaxis (see below). The efficacy of fractalkine was, however, much lower than MCP-1 in monocyte chemotaxis (1.5% versus 30.7%). Among the lymphocyte subpopulations, soluble fractalkine induced efficient migration in $CD16^+$ NK cells and, to a lesser extent, in $CD3^+CD8^+$ T cells (Figure 6B). $CD3^+CD4^+$ T cells also migrated to

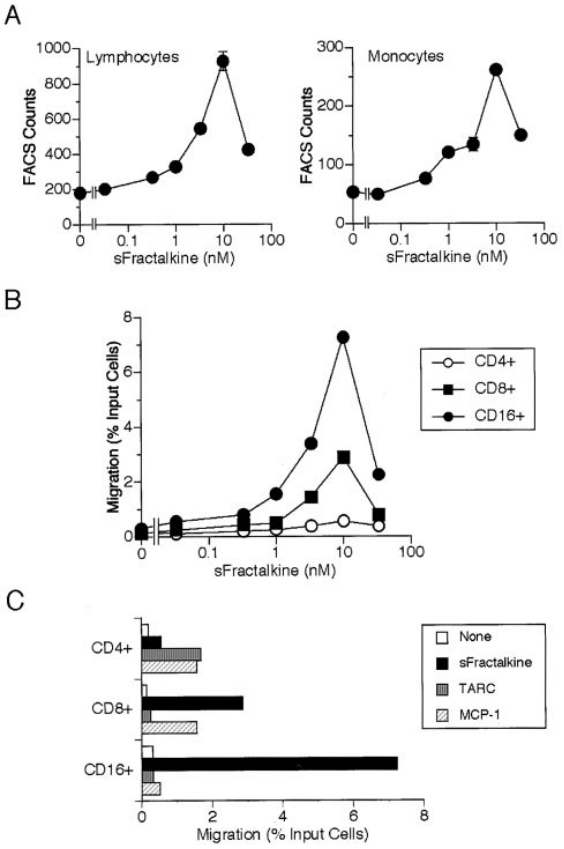

Figure 6. Transendothelial Migration of Human Leukocytes to Soluble Fractalkine

(A) Classes of leukocytes migrating to soluble fractalkine. Cells migrating from upper wells to lower wells were enumerated by counting for 30 sec on a FACStar Plus gating for lymphocytes or monocytes. The data shown represent mean ± range. Representative results from three different donors are shown.

(B) Subsets of lymphocytes migrating to soluble fractalkine. Cells migrated to soluble fractalkine were collected, stained with FITC-conjugated or PE-conjugated antibodies for indicated surface makers, and subjected to flow cytometric analysis. Representative results from three different donors are shown.

(C) Subsets of lymphocytes migrating to soluble fractalkine, TARC, and MCP-1. Cells that migrated to medium (open columns), 10 nM of soluble fractalkine (closed columns), 10 nM of TARC (shaded columns), and 10 nM of MCP-1 (hatched columns) were collected, stained with FITC-conjugated or PE-conjugated antibodies for indicated surface makers, and subjected to flow cytometric analysis. Representative results from three different donors are shown.

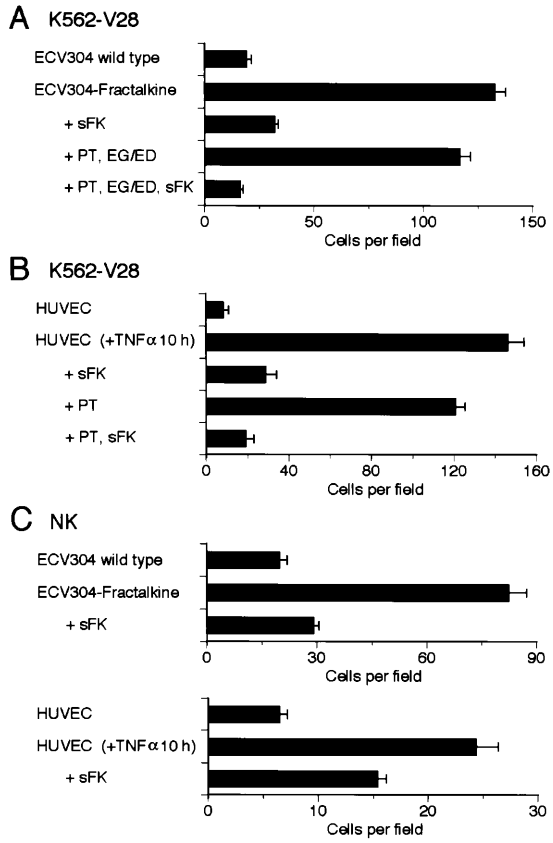

Figure 7. Adhesion of V28-Transfected K562 Cells and Normal NK Cells to Fractalkine-Expressing Endothelial Cells

Control and fractalkine cDNA-transfected ECV304 cells were cultured overnight in 16-well glass slides precoated with polyethyleneimine (A and C). HUVEC were cultured in 16-well glass slides precoated with fibronectin and activated with TNFα (50 ng/nl) for 10 hr (B and C). After the cells in each well were gently washed, K562-V28 (A and B) or normal NK cells (C) were applied to wells and incubated for 20 min at room temperature. After washing off nonadherent cells by dipping the slides gently in assay buffer twice, adherent cells were fixed with 1% glutaraldehyde. For treatment with soluble fractalkine or EGTA/EDTA (EG/ED), 10 μg/ml of soluble fractalkine or 5 mM of EGTA/EDTA was added during the assay. For treatment with PT, cells were pretreated with PT at 500 ng/ml for 1 hr at 37°C. Adhered cells were counted in five 400× fields (0.07 mm^2) per well. The data shown represent mean ± SEM. Representative results from at least two independent experiments are shown.

soluble fractalkine in some individuals at a very low efficiency (<0.6%). Both $CD45RO^+$ memory and $CD45RO^-$ naive cells migrated toward soluble fractalkine (data not shown). No $CD19^+$ B cells migrated to soluble fractalkine at all. We further compared soluble fractalkine with TARC and MCP-1 in the transendothelial migration assay (Figure 6C). $CD3^+CD8^+$ T cells were preferentially attracted by soluble fractalkine, whereas $CD3^+CD4^+$ T cells were preferentially attracted by TARC. Both $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells were equally attracted by MCP-1. However, only soluble fractalkine induced strong migration in $CD16^+$ NK cells. Collectively, the phenotypes of cells migrating to soluble fractalkine correlated well to the subsets of leukocytes expressing *V28* mRNA and fractalkine receptor on the surface.

Adhesion of V28-Expressing Cells to Membrane-Bound Fractalkine on Endothelium

We assessed the role of membrane-bound fractalkine in the adhesion of V28-expressing cells to endothelial cells. As shown in Figure 7A, K562-V28 adhered to ECV304 expressing the membrane form of fractalkine 6-fold more than to control ECV304 cells. Also, consistent with the results of adhesion of K562-V28 to immobilized Fractalkine-SEAP (Figures 3 and 4), soluble fractalkine almost completely suppressed the adhesion, while

PT + EDTA/EGTA had little inhibitory effect on the adhesion. Similar results were obtained when we examined the adhesion of K562-V28 to primary HUVEC. As shown in Figure 7B, K562-V28 cells showed 15-fold more adherence to TNFα-stimulated HUVEC expressing endogenous fractalkine (Bazan et al., 1997) than to control HUVEC. Again, the adhesion of K562-V28 to activated HUVEC was strongly inhibited by soluble fractalkine but only marginally affected by PT. Finally, we examined adhesion of freshly isolated NK cells expressing endogenous V28 to ECV304 and HUVEC (Figure 7C). NK cells adhered to ECV304 expressing fractalkine 4-fold more than to control ECV304. The enhanced adhesion of NK cells to ECV304-fractalkine was strongly inhibited by soluble fractalkine. Similarly, NK cells adhered to TNFα-activated HUVEC 4-fold more than to control HUVEC, and the enhanced adhesion of NK cells to activated HUVEC was partially but significantly inhibited by soluble fractalkine. In the latter case, involvement of other adhesion pathways probably accounted for adhesion not inhibited by soluble fractalkine. Collectively, these results support the conclusion that physical interaction of membrane-bound fractalkine and V28 is indeed capable of directly mediating cell adhesion independent of signaling via the PT-sensitive G proteins or downstream activation of other adhesion molecules, and that this mode of adhesion can be demonstrated in primary human cells as well as cells engineered to express recombinant fractalkine and V28.

Discussion

The CX_3C chemokine fractalkine represents a fourth class of chemokine, which exhibits properties of both traditional chemokines and adhesion molecules, raising questions regarding the molecular nature of its receptor and how the receptor-ligand complex achieves its hybrid functions. We describe here the identification of the 7-TM protein encoded by the *V28* orphan cDNA as a specific, high-affinity receptor for fractalkine. Since the V28 protein represents the first receptor for a CX_3C chemokine, we have accordingly designated it as CX_3CR1. As in the case for CC and CXC chemokine receptors, CX_3CR1 is a 7-TM G protein–coupled receptor (Combadiere et al., 1995; Raport et al., 1995). CX_3CR1 contains several motifs conserved among the chemokine receptor superfamily. For example, a DRY motif, which is important for G protein interactions and signal transduction, is present. We showed in the present study that calcium mobilization and cell migration responses linked to CX_3CR1 were highly sensitive to PT (Figure 2), indicating coupling to the Gαi class of G proteins. These results suggest that, at least in transfected cells, CX_3CR1 is capable of interacting with signal transduction machinery shared with other CCRs and CXCRs (Campbell et al., 1996). It may also be noteworthy that, when aligned phylogenetically with other chemokine receptors, CX_3CR1 is more closely related to CC chemokine receptors than to those for CXC chemokines. This may relate to its biological specificity for T cells, NK cells, and monocytes, which mimic the known activities of CC chemokines (Baggiolini et al., 1994; Rollins, 1997).

The previous study demonstrated the ability of fractalkine to support the adhesion of monocytes and T cells when expressed on HEK 293 cells (Bazan et al., 1997). However, that report did not address whether fractalkine itself was an adhesion molecule or whether it was merely inducing and/or activating other adhesion molecules on the surface of HEK 293 cells, or leukocytes, or both. The involvement of other adhesion molecules was an especially compelling notion since the mucin domain of fractalkine is reminiscent of structures seen elsewhere in the realm of adhesion molecules. For example, L-selectin binds to the carbohydrate determinants exposed on the mucin structure of MAdCAM-1, GlyCAM-1, or CD34 (Girard and Springer, 1995). Nevertheless, the identification of the CX_3CR1 has led to findings that do not support this model. In addition, the involvement of other adhesion molecules seems also precluded. Several lines of evidence in the present study support this view. First, CX_3CR1-expressing cells efficiently adhere to purified fractalkine immobilized onto glass slides, showing that the adhesion can occur in the absence of substrates for other cell adhesion molecules (Figure 3). Second, the adhesion was robust in the absence of divalent cations, which are required for integrin- or selectin-mediated adhesion (Figure 3) (Springer, 1994). We also confirmed that 293/EBNA-1 cells do not express CD18, the essential subunit of β2 integrins, and K562 cells do not express VLA-4 (data not shown). Furthermore, neutralizing monoclonal anti-CD18, anti-LFA-1, anti-CD29, anti-CD49d, and anti-CD44 had no inhibitory effect on the adhesion (data not shown). Taken together, the evidence suggests that fractalkine and CX_3CR1 are molecules capable of directly mediating cell adhesion as well as chemotaxis.

Fractalkine and the CX_3CR1 mediate the processes of chemotaxis and adhesion directly, but the machinery required for each activity is distinct. The migratory and calcium mobilization functions controlled by CX_3CR1 are G protein–linked and can be inhibited by PT (Figures 2 and 3). By contrast, adhesion occurs in all CX_3CR1 transfected cell lines tested, even in those that do not mobilize calcium to fractalkine or in those that are not chemotactically responsive to soluble fractalkine (Figure 3). Adhesion to fractalkine is also insensitive to PT (Figure 3). Thus, the adhesion via fractalkine and CX_3CR1 occurs before, and certainly appears to be independent of, activation of integrins and chemotaxis. This, however, does not exclude the role of CX_3CR1 signaling via the Gαi class of G proteins in activation of integrins for subsequent firm adhesion. Another notable point is that the adhesion mediated by CX_3CR1 and fractalkine is dependent on temperature (Figure 3). This may suggest involvement of some signaling via CX_3CR1 in the observed adhesion. It is, however, more likely that membrane fluidity is needed for CX_3CR1-expressing cells to form multiple associations with immobilized fractalkine at levels sufficient for cell adhesion.

These results prompted our molecular dissection of how the fractalkine architecture was important in the achievement of adhesion with CX_3CR1. Fractalkine is apparently a hybrid molecule comprising the CX_3C chemokine as an effector domain and the mucin-like stalk as a presentation structure (Bazan et al., 1997). First, the binding of fractalkine to CX_3CR1 seems to

be solely the function of the chemokine domain. The chemokine domain alone bound to CX_3CR1 as efficiently as the whole extracellular region of fractalkine, giving a similar high affinity and number of binding sites (Figure 4). With regard to receptor binding, the mucin domain seemed to have no direct role, since the mucin domain alone showed no binding to CX_3CR1-expressing cells nor any positive or negative effect on the binding of the chemokine domain to CX_3CR1 (Figure 4). Collectively, these results indicate that the chemokine domain is the sole effector domain for CX_3CR1, possessing a similar biological activity as other CC, CXC, or C chemokines.

What, then, is the role of the mucin-like domain? We demonstrated that both the chemokine and mucin domains were necessary for efficient adhesion induction of CX_3CR1-expressing cells (Figure 4). However, the soluble mucin domain alone or the mucin domain linked to a CC chemokine TARC did not inhibit cell adhesion mediated by immobilized fractalkine. Furthermore, coimmobilization of the chemokine domain and the mucin domain as separate molecules at a 1:1 ratio did not support the adhesion at all (Figure 4). Thus, it seems unlikely that the mucin domain is directly involved in cell adhesion as an adhesion domain. Most probably, the mucin domain functions primarily as an efficient presentation molecule for the chemokine domain. This, however, does not exclude a potential role of the mucin domain in cell adhesion as a ligand for some lectins in natural settings.

In the case of other CC, CXC, or C chemokines, they have to be retained and presented by surface molecules on endothelial cells, otherwise they are rapidly diluted by blood flow (for review, see Schall and Bacon, 1994). The heparin-binding property of these chemokines may provide a mechanism for retention in the extracellular matrix or by cell-bound proteoglycan so as to be presented on the endothelial cells for rolling lymphocytes. Fractalkine, possessing intrinsically both the chemokine and presentation domains, may represent a parsimonious solution to the presentation problem. Consistent with this idea, even a CC chemokine, TARC, if linked to the mucin domain of fractalkine, was capable of inducing efficient adhesion of cells expressing its cognate receptor, CCR4 (Imai et al., 1997) (Figure 4). This observation suggests that other chemokine receptors also have a potential to support adhesion as CX_3CR1 if chemokines are presented in a structure similar to fractalkine. This may lead to a hypothesis that chemokines and their receptors, if the former are presented on molecules such as proteoglycans, are capable of directly mediating adhesion of leukocytes as well as signaling for downstream activation of integrins during the multistep process of leukocyte trafficking (Butcher, 1991; Springer, 1994; Butcher and Picker, 1996).

We demonstrated that soluble fractalkine induces migration of certain types of leukocytes through endothelial cells in a transendothelial migration assay. In accordance with the expression of *CX_3CR1* mRNA and surface fractalkine receptor (Figure 5), $CD16^+$ NK cells and $CD3^+CD8^+$ T cells efficiently migrated toward soluble fractalkine (Figure 6). Approximately 92% of $CD16^+$ NK cells and 25% of $CD3^+CD8^+$ T cells express fractalkine receptor on the cell surface (Figure 5), and 8% of $CD16^+$ NK cells and 2.5% of $CD8^+$ T cells added to the assay migrated (Figure 6). Thus, roughly 10% of the receptor-expressing cells of each subset responded to soluble fractalkine in our experimental conditions. Although 80% of $CD14^+$ monocytes expressed fractalkine receptors (Figure 5), only 1% of input cells, that is, only 1.3% of the receptor-expressing cells, migrated to soluble fractalkine (Figure 7). Furthermore, THP-1 cells, a monocytic cell line, migrated to MCP-1 but not to soluble fractalkine, even though they expressed fractalkine receptor on the surface (data not shown). These results suggest that the receptor expression per se does not determine migration activity of leukocytes to fractalkine.

Fractalkine is likely to be expressed on the cell surface of endothelial cells in inflamed tissues (Bazan et al., 1997). This would induce adhesion of cells such as NK cells expressing CX_3CR1. To address this notion, we examined the role of fractalkine in the adhesion of CX_3CR1-expressing cells to fractalkine-expressing endothelial cells. K562 cells expressing CX_3CR1 vigorously adhered to ECV304 cells expressing transfected fractalkine and to TNFα-stimulated HUVEC expressing endogenous fractalkine (Figure 7). The enhanced adhesion of K562 cells expressing CX_3CR1 to fractalkine-expressing cells was almost completely blocked by soluble fractalkine (Figure 7), even though soluble fractalkine itself was a potent agonist for CX_3CR1 in induction of chemotaxis and calcium mobilization (Figure 3). Furthermore, PT or EDTA/EGTA had little inhibitory effect on the observed cell adhesion. Thus, the cell-to-cell adhesion promoted by CX_3CR1 and membrane-bound fractalkine was also mostly independent of signaling via PT-sensitive G proteins or divalent cation-dependent integrins and selectins, and thus probably mediated by direct physical association of fractalkine and CX_3CR1. Similarly, freshly isolated NK cells expressing endogenous CX_3CR1 (Figure 5) adhered well to ECV304 cells expressing transfected fractalkine and also to TNFα-treated primary HUVEC expressing endogenous fractalkine (Figure 7). The adhesion of NK cells to these types of cells was also effectively blocked by soluble fractalkine (Figure 7). Collectively, the membrane form of fractalkine expressed on activated endothelial cells indeed induced adhesion of CX_3CR1-expressing cells such as NK cells. The precise role of fractalkine in interaction of CX_3CR1-expressing cells and activated endothelial cells, however, requires further analysis in more physiological flow conditions.

In summary, the identification of CX_3CR1 illuminates unique features in the molecular control of leukocyte trafficking. At the critical interface between leukocyte cell surface and endothelium, fractalkine and CX_3CR1 seem to blend adhesion and chemotactic properties at the molecular and functional levels. Aside from enhancing the basic understanding of leukocyte migration, these molecules may also define new targets for development of antiinflammatory agents.

Experimental Procedures

Cells

Cells stably expressing CCR1, CCR2B, CCR3, CCR4, CCR5, CCR6, CCR7, V28/CMKBRL1, BLR1, or GPR-9–6 (GenBank accession number: HSU45982) were described previously (Imai et al., 1997).

ECV304 cells were obtained from American Type Culture Collection and maintained in M199 medium supplemented with 10% FBS. For stable expression of membrane-bound fractalkine in ECV304 cells, the expression plasmid pCAGG-Neo-fractalkine-1 was transfected into ECV304 by Lipofectamine (GIBCO-BRL). After selection with 800 μg/ml of G418 for 1–2 weeks, drug-resistant cells were pooled and incubated with an anti-fractalkine MAb (T. I. et al., unpublished data). After washing, cells were stained with FITC-conjugated anti-mouse IgG, and cells expressing membrane-bound fractalkine were obtained by sorting on a FACStar Plus. Peripheral blood mononuclear cells (PBMC) and granulocytes were isolated from venous blood obtained from healthy adult donors as described previously (Imai et al., 1997). Further fractionation of lymphocytes into classes and subsets was also carried out as described previously (Imai et al., 1997). The purity of each cell population was in the range of 95%–99% as determined by flow cytometric analysis.

Production of Recombinant Proteins

To express Fractalkine-SEAP, the DNA fragment encoding fractalkine was amplified from fractalkine cDNA by PCR using 5′ SalI-fractalkine primer (+5′-CGCGTCGACTCAGCCATGGCTCCGATA TCT-3′) and 3′ fractalkine-XbaI primer (−5′-CGCTCTAGAGGTGGC AGCCTGGGCGTCAGG-3′) and subcloned into pDREF-SEAP$(His)_6$-Hyg vector as described previously (Imai et al., 1997). CX_3C-SEAP was similarly generated using 5′ SalI-fractalkine primer (see above) and 3′ fractalkine-XbaI-2 primer (−5′-CGCTCTAGATAGGGCAGCA GCCTGGCGGTC-3′). For generation of Mucin-SEAP, the DNA fragment encoding SalI-oncostatin M signal sequence-XbaI-fractalkine lacking the chemokine domain NheI was amplified by three-step PCR using 5′ SalI-OMC-XbaI-fractalkine primer (+5′-CTGTTTCCA AGCATGGCGAGCATGTCTAGAAATGGCGGCACCTTCGAGAAG-3′) and 3′ fractalkine-NheI primer (−5′-CGCGCTAGCGGTGGCAGCCT GGGCGTCAGG-3′). TARC-SEAP was described previously (Imai et al., 1997). TARC-mucin-SEAP was generated by replacing oncostatin signal sequence of Mucin-SEAP to TARC. 293/EBNA-1 cells were transfected with the expression vectors for various recombinant proteins by using Lipofectamine (BRL). After 3–4 days, the supernatants were collected. For one-step affinity purification, supernatants were applied to 1 ml of Hisbond resin (Qiagen, Hilden, Germany). After washing, bound recombinant proteins were eluted with 100 mM imidazol. The concentration of each recombinant protein was determined by a sandwich-type enzyme-linked immunosorbent assay as described previously (Imai et al., 1997). AP activity was determined by a chemiluminescent assay using Great Escape Detection Kit (Clontech). The enzymatic activity was expressed as relative light unit (RLUs).

Soluble fractalkine with a tag of six histidine residues, $(His)_6$, at its C terminus was prepared by using a baculovirus expression system. The cDNA fragment encoding the extracellular domain of fractalkine was subcloned into the SalI-XbaI sites of the modified pFastBac1 baculovirus transfer vector (GIBCO-BRL) to express fractalkine as a soluble fusion protein with Ser-Arg-Ser-Ser-Gly-$(His)_6$. The recombinant bacmids were generated in E. coli DH10Bac and transfected into Spodoptera frugiperda Sf9 cells using Lipofectin (GIBCO-BRL) to obtain the recombinant viruses. For expression of the recombinant fractalkine-$(His)_6$, Trichoplusia ni BTI-TN-5B1–4 cells were infected with the recombinant viruses at moi of 10–20. The culture supernatants collected 2 days after infection were applied to a 1 ml Hisbond resin (Qiagen). After washing, bound fractalkine-$(His)_6$ was eluted with 100 mM imidazol. Fractions containing recombinant fractalkine-$(His)_6$ were pooled and dialyzed to PBS. Protein concentrations were determined by the BCA kit (Pierce, Rockford, IL). Endotoxin levels were determined by the Limulus amoebocyte lysate assay (QCL-1000) (Bio Whitaker, Walkersville, MD) and were <4 pg per μg of recombinant fractalkine-$(His)_6$.

Receptor Binding Assay

This was carried out as described previously (Imai et al., 1997). In brief, cells were incubated for 1 hr at 16°C with increasing concentrations of SEAP-fusion proteins in the presence or absence of 200 nM soluble fractalkine in 200 μl of RPMI-1640 containing 20 mM HEPES (pH 7.4), 1% BSA, and 0.02% sodium azide. After incubation, cells were washed, lysed in 50 μl of 10 mM Tris-HCl (pH 8.0)/1% Triton X-100, and heated at 65°C for 10 min to inactivate cellular phosphatases. Lysates were collected by centrifugation, and AP activity in 10 μl of lysate was determined by the chemiluminescent assay. All assays were done in duplicate. Binding data were analyzed by the LIGAND program.

Chemotaxis and Calcium Mobilization Assays

Cell migration assay in a 48-well microchemotaxis chamber and across a polyvinylpyrrolidone-free polycarbonate filter (8 μm pores) precoated with type IV collagen was carried out as described previously (Imai et al., 1997). Each sample was assayed in triplicate, and migrated cells were counted in five randomly selected high-power fields (×400) per well. Calcium mobilization assay was carried out as described previously (Imai et al., 1997). Transendothelial chemotaxis assay was carried out by using an endothelial cell line ECV304 as described previously (Carr et al., 1994; Oin et al., 1996). In brief, ECV304 cells (2×10^5) were added on Transwell culture inserts (Coaster) with a 5 μm pore size and cultured at 37°C for 48–96 hr in M199 medium supplemented with 10% FBS. Chemokines were diluted in migration medium (RPMI-1640:M199=1:1, 0.5% BSA) and added to 24-well tissue culture plates in a final volume of 600 μl. Endothelial cell–coated inserts were placed in each well, and 10^6 PBMC in 100 μl were added to the upper chamber. The cells were allowed to migrate through endothelial cells to the lower chamber at 37°C for 4 hr. The cell suspensions from the lower chamber were then stained with FITC or PE-conjugated MAb to CD3, CD4, CD8, CD14, CD16, or CD19. The migrated cells were then counted using flow cytometry.

Adhesion Assay

Each well of 16-well glass slides was coated with 50 μl of anti-SEAP antibody (10 μg/ml) in 50 mM Tris-HCl (pH 9.5) at 4°C overnight. After washing with PBS, nonspecific binding sites were blocked with adhesion buffer (RPMI-1640, 1% BSA, 20 mM HEPES [pH 7.4]). The SEAP fusion proteins (10 nM) were added to wells, and the plates were incubated at room temperature for 2 hr and washed extensively. Cells (4000 cells/mm^2) were applied to each well in final volume at 50 μl and incubated for 30 min at room temperature. After washing off nonadherent cells by dipping the slides gently in adhesion buffer twice, cells were fixed with 1% glutaraldehyde. To assess cell adhesion to endothelial cells, control ECV304 and ECV304 transfected with the full-length fractalkine cDNA were added to wells precoated with polyethyleneimine at 1×10^5 cells per well a day before the assay. Similarly, HUVEC were added to wells precoated with fibronectin at 2×10^4 cells per well and cultured for 2 days before the assay. Some HUVEC cultures were treated with TNFα for 10 hr prior to the assay. The purity of NK cells used was in the range of 80%–90% as determined by flow cytometric analysis. All assays were performed in duplicate, and the percent adhesion of input cells was determined by counting cells in five 400× fields (0.07 mm^2) per well.

Northern Blot and Flow Cytometric Analysis

Northern blot analysis was carried out as described previously (Imai et al., 1997). For flow cytometric analysis of fractalkine receptor, PBMC or granulocytes were incubated with 10 nM of Fractalkine-SEAP or control SEAP on ice for 30 min in staining buffer (1% FBS, 2.5% human AB serum, 0.02% sodium azide in PBS). After washing, cells were incubated with biotinylated anti-SEAP antibody, washed, and then incubated with FITC-conjugated streptavidin and PE-conjugated antibody for leukocyte subset makers. Cells were then analyzed on a FACStar Plus (Beckton Dickinson, Mountain View, CA).

Acknowledgments

We thank Drs. Y. Himuna, M. Hatanaka, K. Takatsuki, and R. Miura for constant support and encouragement.

Received May 23, 1997; revised October 6, 1997.

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